



Wound response in orange as a resistance mechanism against *Penicillium digitatum* (pathogen) and *P. expansum* (non-host pathogen)

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ABSTRACT

Penicillium digitatum is the most devastating postharvest pathogen of citrus. In addition, *Penicillium expansum* is the main pathogen of pome fruit, although recent studies have demonstrated its ability to infect oranges under some conditions. In this study, we evaluated wound response in 'Valencia' oranges harvested at three different maturity stages and the effect of wound response on the establishment of both pathogens when fruit were stored at two different temperatures (20 and 4 °C). The effect of wounding and pathogen inoculation on lignin content, was also quantified. Lastly, the expression of several phenylpropanoid pathway-related genes was also analyzed by semi-quantitative RT-PCR. Results indicated that, in general, *P. digitatum* exhibited lower decay incidence and severity as time between wounding and inoculation increased. Decay incidence and severity were higher in fruit from the over-mature harvest than in fruit from immature and commercial harvests. *P. expansum* was able to infect fruit at 20 °C but lesions were small compared to lesion size of fruit stored at 4 °C. Lignin content in wounded fruit (control) and in samples wounded and inoculated with *P. expansum* was highest in fruit from the immature harvest at 7 d post-wounding and inoculation. Wounded fruit had higher expression of *pal1*, *comt1* and *pox1* genes at 48 h than at 24 h. However, samples inoculated with *P. digitatum* showed lower expression at 48 h than at 24 h. Our results indicated that maturity and storage temperature play an important role in orange wound response.

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1. Introduction

Penicillium digitatum is a major postharvest pathogen of citrus while blue mould, caused by *Penicillium expansum*, is a devastating postharvest pathogen of pome fruit. While these pathogens are usually host specific, under some conditions, *P. expansum* is able to infect oranges (Vilanova et al., 2012). Both pathogens are necrotrophs and require a wound in the epidermis to enter fruit tissue and initiate infection (Kavanagh and Wood, 1967; Spotts et al., 1998). Since conidia of *Penicillium* species are ubiquitous in the atmosphere of packinghouses (Barkai-Golan, 1966), fruit infection can occur via injuries caused during harvest, transport, packing-house manipulation, or storage. Therefore, good sanitation and handling practices in both the field and packinghouse are critical. In addition to these preventive actions, drenching and fogging treatments with chemical fungicides represent the main method used to control these fungi. The use of chemical fungicides, however, is

becoming increasingly more restricted because of environmental and health concerns, as well as due to the development of fungal resistance. New approaches, based on the innate resistance of the fruit, need to be explored in order to reduce the use of chemical fungicides so that they can be applied only when strictly necessary (Ballester et al., 2010).

Wounding is a common occurrence in plants resulting from both abiotic factors such as wind, rain and hail, as well as biotic factors such as insect and herbivore feeding, and in the case of agricultural commodities, cultural manipulation (Cheong et al., 2002). Plants exhibit a variety of defence strategies in response to wounding in order to prevent pathogen invasion. In fruit tissues, these wound-induced defence responses may be modulated by ripening (Su et al., 2011). Wound responses in plants have been extensively studied (Leon et al., 2001; Schillmiller and Howe, 2005) and it has been hypothesized that plants have evolved mechanisms that integrate both pathogen-specific and general wounding responses (Castro-Mercado et al., 2009). In support of this idea, studies have shown that wounding regulates a number of genes that are associated with a pathogen-specific response (Durrant et al., 2000; Reymond et al., 2000), indicating that innate and pathogen-specific responses share

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a number of components in their signalling pathways (Maleck and Dietrich, 1999).

The initial stages of a plant's response to an invading pathogen will determine the degree of colonization and the extent of damage (Gayoso et al., 2010). Limiting pathogen establishment and colonization depends on a rapid and efficient deployment of defence responses (Ferreira et al., 2006) which will be modulated depending on whether the host-pathogen interaction involves a compatible or incompatible pathogen. Recognition of a pathogen may lead to the activation of defence mechanisms, such as a hypersensitive response, an oxidative burst, and the upregulation of pathogenesis-related (PR) genes (Albrecht and Bowman, 2008).

An oxidative burst is a rapid generation of reactive oxygen species (ROS) and is one of the earliest events that widely occurs during a plant-pathogen interaction. It has been implicated in many different processes related to host-pathogen interactions (Shetty et al., 2008) and also plays an important role in wound response (Bradley et al., 1992). ROS production has been associated with the formation of physical defensive barriers in plant cell walls (Huckelhoven and Kogel, 2003) involving the formation of glycoproteins, callose, lignin, and other phenolic polymers (Lamb and Dixon, 1997).

The presence of lignin in plant tissue is recognized as a key factor in disease resistance to infections, serving as a strong mechanical barrier against pathogen invasion (Friend, 1976). Lignification occurs through a series of enzymatic steps involving the phenylpropanoid pathway, a pathway that generally contributes to a variety of plant responses to biotic and abiotic stimuli (Vogt, 2010). Phenylalanine ammonia-lyase (PAL) is the first enzyme in the phenylpropanoid pathway (Olson and Varner, 1993) leading to the synthesis of coumarins and flavonoids (Dixon et al., 2002). Ballester et al. (2011), in a study of citrus, focused on changes in PAL expression in response to a compatible pathogen (*P. digitatum*). They demonstrated that in addition to PAL, a large subset of genes are involved in the synthesis of phenylpropanoids and flavonoids, such as caffeic acid O-methyl-transferase (COMT), cinnamyl alcohol dehydrogenase (CAD), sinapyl alcohol dehydrogenase (SAD) and also peroxidase (POX), a terminal enzyme involved in the polymerization of lignin.

The aim of the present study was to investigate the process of wound response in citrus to both compatible, *P. digitatum*, and non-host, *P. expansum*, pathogen at different (i) maturity stages; and (ii) storage temperatures. Lignin content, as well as the expression of several genes involved in the phenylpropanoid pathway, were quantified to define their role in host resistance against both pathogens.

2. Materials and methods

2.1. Fruit

'Valencia' oranges (*Citrus sinensis* L. Osbeck) were obtained from a commercial orchard in Tortosa (Catalonia, Spain) and used immediately after harvest. Harvests were carried out on the 20th March (harvest 1), 30th April (harvest 2) and 29th June (harvest 3), 2010. Harvest 1 was considered as prior to commercial maturity (immature harvest), harvest 2 was considered commercial maturity (commercial harvest) and harvest 3 was considered past maturity (over-mature harvest). Fruit were selected for uniform size, without physical injuries or apparent infections. Once the oranges arrived at the laboratory, they were surface disinfected with 10% sodium hypochlorite for 1 min, rinsed with tap water, and allowed to dry at room temperature. Colour, firmness, soluble solids, and acidity were determined as quality parameters at each harvest date.

2.2. Determination of quality parameters

Colour was measured on opposite sides of each fruit using a tri-stimulus colourimeter (Chromameter CR-200, Minolta, Japan). The mean values for the lightness (L^*), red-greenness (a^*) and yellow-blueness (b^*) parameters were calculated for each fruit and expressed as a Colour index ($CI = (1000 \cdot a^*) / (L^* \cdot b^*)$). Firmness measurements were performed using a TA-XT2i Texture Analyser (Stable Micro Systems Ltd., Surrey, UK), based on the millimetres of fruit deformation resulting from fruit response to 2 kg of pressure on the longitudinal axis at a constant speed of 2 mm s^{-1} . Total soluble solids (TSS) and titratable acidity (TA) were assessed in extracted juice using a refractometer (Atago, Tokyo, Japan), and by titration of 10 mL of juice with 0.1 N NaOH and 1% phenolphthalein as an indicator, respectively. Data on maturity indices represent the means of 20 individual fruit. Maturity index was calculated as a ratio of TSS/TA.

2.3. Fungal cultures

P. digitatum (PDM-1) and *P. expansum* (CMP-1) are the most aggressive isolates in our collection of isolates capable of infecting citrus and pome fruit, respectively. They are maintained on potato dextrose agar medium (PDA; 200 mL boiled potato extract, 20 g dextrose, 20 g agar and 800 mL water) and periodically grown on wounded oranges (*P. digitatum*) or apples (*P. expansum*) and then re-isolated to maintain virulence. Conidial suspensions were prepared by adding 10 mL of sterile water with 0.01% (w/v) Tween-80 over the surface of 7- to 10-day-old cultures grown on PDA and rubbing the surface of the agar with a sterile glass rod. Conidia were counted in a haemocytometer and diluted to the desired concentration.

2.4. Wound response studies

The effect of maturity and storage temperature on wound response was assessed for both the compatible interaction (*P. digitatum*-oranges) and the incompatible interaction (*P. expansum*-oranges). Oranges were wounded once with a nail (1 mm wide and 2 mm deep). To evaluate the effect of storage temperature on wound response, fruit were separated in two different sets; one was stored at 20°C and the other at 4°C .

Fruit stored at 20°C were divided into 7 different subgroups, each one inoculated at different times after wounding: time 0 h (wounded and inoculated at the same time) served as a control while the other 6 subgroups were inoculated at 1, 2, 3, 4, 7 or 10 d after wounding. The experiment was carried out for each pathogen and at each maturity stage. In all cases, fruit were inoculated with $15 \mu\text{L}$ aqueous conidia suspensions of *P. digitatum* at 10^5 conidia mL^{-1} and *P. expansum* at 10^7 conidia mL^{-1} . Incidence and severity of lesions were evaluated after 4, 7 and 10 d of inoculation for each pathogen, time between wounding and inoculation, and maturity stage.

Fruit stored at 4°C were divided into 5 different subgroups, each one inoculated at different times after wounding: time 0 h (wounded and inoculated at the same time) served as a control while the other 4 subgroups were inoculated at 4, 7, 14 or 21 d after wounding. Fruit were inoculated as previously described and the experiment was carried out for each pathogen and maturity stage. Incidence and severity of lesions were evaluated at 30, 45 and 60 d after inoculation for each pathogen, time between wounding and inoculation, and maturity stage.

In both cases (20°C and 4°C), five oranges constituted a single replicate and each treatment was repeated four times.

2.5. Lignin studies

Lignin content of oranges was measured at three different maturity stages and at four times after inoculation (24, 48, 96 h, and 7 d) for both pathogens.

To measure the lignin content, thirty-five wounds were made on one side of each orange with a nail in a manner similar to in the wound response studies, and inoculated with 10 μ L of aqueous conidial suspensions of either *P. digitatum* or *P. expansum* at 10^7 conidia mL^{-1} . Control fruit were wounded and inoculated with 0.01% (w/v) Tween-80 (control). Fruit were stored at 20 °C and 85% RH for 24, 48, 96 h, and 7 d.

After each storage time, 30 cylinders of peel tissue (5 mm inside diameter and 4 mm deep with flavedo and albedo) encompassing the wounds were removed from each orange using a cork borer. Ninety disks from three fruit were pooled and considered a biological replicate and three biological replicates were evaluated for each sample collection.

The estimation of lignin content was performed according to Nafussi et al. (2001) with slight modification. Briefly, frozen peel disks were lyophilized for 3 d and then ground to a fine powder. Each sample was sequentially washed with water, ethanol, acetone and diethyl ether through Whatman 1 filter paper until the washed tissue was colourless. The resulting powder was dried at 70 °C for 1 h, and 20 mg samples were digested with a solution of 25% (w/w) acetyl bromide in acetic acid (2.5 mL) and HClO_4 (70%, 0.12 mL) and heated in a bath at 70 °C for 30 min with shaking. After cooling with ice, 10 mL of 2 M NaOH and 12 mL of acetic acid were added to the reaction tubes and 1.5 mL of the resulting solution was centrifuged at $14,000 \times g$ (Mikro 22R, Hettich Zentrifugen, UK) for 11 min at room temperature to be sure that the resulting sample was completely clear. Each solution was diluted 5 times with acetic acid and absorbance was measured at 280 nm. For each replicate, three technical measurements were done.

2.6. Gene expression analysis

Semi-quantitative reverse transcription (RT-PCR) approach was used to estimate relative mRNA levels of several genes involved in the phenylpropanoid pathway (Table 1) in response to wounding and wounding plus inoculation with either *P. digitatum* or *P. expansum*. As described previously, each orange received 35 wounds on one side of each fruit using a nail and inoculated with 10 μ L aqueous conidia suspensions of *P. digitatum* or *P. expansum* at 10^7 conidia mL^{-1} concentration. Control fruit were wounded and inoculated with 0.01% (w/v) Tween-80 (control). Fruit were stored at 20 °C and 85% RH for 24 and 48 h. The procedure used to collect tissue samples was the same used in the lignin assay. Three hundred disks from ten fruit were pooled and considered a biological replicate and each sample collection consisted of three biological

replicates. All samples were immediately ground in liquid nitrogen using a mortar and pestle and stored at -80°C until further processing.

Total RNA was isolated as described by Ballester et al. (2006) with slight modifications. Briefly, 2 g of ground orange peel tissue were added to a 65 °C mixture of 10 mL of extraction buffer (200 mM Tris-HCl, pH 8.0, 400 mM NaCl, 50 mM EDTA, 2% (w/v) sodium-*n*-lauroylsarcosine (SDS), 1% (w/v) polyvinyl-pyrrolidone 40, 1% β -mercaptoethanol) and 5 mL of Tris-equilibrated phenol. After homogenization with a Polytron PT 45/80 (Kinematica AG, Lucerne, Switzerland) for 1 min, the extract was incubated at 65 °C for 15 min and cooled before the addition of 5 mL of chloroform:isoamyl alcohol (24:1, v/v). The homogenate was centrifuged at $3170 \times g$ (Avanti J-20XP, Beckman Coulter) for 20 min at 4 °C and the aqueous phase was re-extracted with 10 mL of phenol:chloroform:isoamyl alcohol (25:24:1, v/v/v) and centrifuged at $3170 \times g$ for 15 min at 4 °C. Nucleic acids were precipitated overnight at -20°C by adding 1/3 volume of 12 M lithium chloride in a 40 mL centrifuge tubes. After centrifugation at $9552 \times g$ for 60 min at 4 °C, the precipitate was incubated with 500 μ L of 3 M sodium acetate, pH 5.2, for 15 min at -20°C and centrifuged at $14,160 \times g$ for 5 min at room temperature to remove residual polysaccharides. The resulting pellet was washed with 500 μ L of 70% cold ethanol and centrifuged immediately at $14,160 \times g$ for 5 min at room temperature. The pellet was dissolved in 50 μ L of sterile water and heated at 65 °C for 10 min. Finally, insoluble material was discarded after centrifugation at $14,160 \times g$ for 5 min at room temperature. RNA concentration was measured using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE, USA) and its integrity was verified by agarose gel electrophoresis using 1 \times sodium-boric (SB) acid as running buffer (Brody and Kern, 2004). RNA was visualized by staining with GelRed™ Nucleic Acid Gel Stain (Biotium, Hayward, CA, USA).

Extracted RNA was treated and purified with rDnase Set and NucleosSpin® RNA Clean-up XS (Macherey-Nagel, Düren, Germany) according to the protocol provided by the manufacturer. Aliquots of 5 μ g total RNA were used for first-strand cDNA synthesis in 20 μ L reactions with 200 U SuperScript™ III RT (Invitrogen, Carlsbad, CA, USA).

Semiquantitative-PCR amplifications were done in a total volume of 60 μ L containing 6 μ L 10 \times Buffer BioTaq (Bioline, London, UK), 1.5 mM MgCl_2 , 1 unit BioTaq DNA polymerase (Bioline, London, UK), 0.2 mM dNTP, 0.4 μ M primer (Invitrogen, Madrid, Spain) and a cDNA amount equivalent to 120 ng of RNA. DNA amplification was carried out in a thermal cycler GeneAmp® PCR System 2700 (Applied Biosystem, Madrid, Spain) with the following programme of an initial denaturalization step of 94 °C for 5 min, and 31 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 40 s, followed by a final extension step of 72 °C for 7 min. Aliquots of 9 μ L were removed after 21, 23, 25, 27, 29 and 31 cycles and analyzed by

Table 1

Primer sequences, expected PCR product sizes (bp) and optimal annealing temperatures (°C) used to analyze expression of several phenylpropanoid pathway-related genes in orange.

Name	Sequence	Gene	GenBank accession no.	Annealing T (°C)	Amplicon (bp)
PAL F1 PAL R1	AGGCAGACTGTTGAGAATGGA CCATTACTACATCGCAAT	Phenylalanine ammonia lyase 1	AJ238753	60	287
COMT1 F1 COMT1 R1	CACAGTTTGGCCAATGGTTCT GCCATAAGGCTCTCAACTCC	Caffeic acid O-methyltransferase 1	FC924158	60	235
CAD2 F1 CAD 2 R1	AAAGGCTATGGGGTTAAGGT CGGGTGAAGCTGCAGATACTGT	Cinnamyl alcohol dehydrogenase 2	CX298708	60	172
POX1 F1 POX1 R1	AGGGTGATTGGTCAGCTCTT AGCAGGACGAGACACAAAAA	Peroxidase 1	CX302828	60	228
SAD F1 SAD R1	ATGCATTCTTGTTCACAGTG TCATTCTCCAATGTGCTTC	Sinapyl alcohol dehydrogenase	GR312966	60	231

electrophoresis on 1.3% agarose gel with 1× SB buffer. Standard DNA samples (1 kb Plus DNA Ladder, Invitrogen, Madrid, Spain) were used as molecular size marker.

The primer pairs and annealing temperature for each gene are listed in Table 1. The primer pairs were based on gene sequence reported by Ballester et al. (2011). In order to select a putative housekeeping gene, several different genes (Table 2) were analyzed following the same methodology described above. Additionally, the selected housekeeping gene was used to confirm the absence of genomic DNA contamination.

2.7. Data analysis

Data regarding incidence and severity of decayed fruit, lignin content and quality parameters were analyzed for significant differences by analysis of variance (ANOVA) with JMP 8 (SAS Institute Inc., NC, USA) statistical package. Before analysis of data expressed as percentages, homogeneity of variance was tested by Barlett's test and data were transformed to the arcsine of the square root. Statistical significance was deemed when $P < 0.05$. When the analysis was statistically significant, a Tukey test for separation of means was performed.

3. Results

3.1. Effect of maturity stage and time between wounding and inoculation on development of green mould caused by *P. digitatum*

In general, the elapsed time between wounding and inoculation had a significant effect on restricting *P. digitatum* infection and the effect was more pronounced in fruit from the immature and commercial harvests compared to the over-mature harvest. The overall response observed at 4 and 20 °C were similar.

3.1.1. At 20 °C

Decay incidence and lesion diameters in 'Valencia' oranges inoculated with *P. digitatum* at different times after wounding and incubated at 20 °C are shown (Fig. 1A and B). Data represents observations at 7 d following inoculation. In general, little evidence of decay was present at 4 d after inoculation (data not shown) and at 10 d after inoculation, differences among times between wounding and inoculation were difficult to evaluate because of the extensive rots (15 cm lesion diameter) that were present (data not shown).

At immature harvest, decay incidence during the first 2 d following wounding was nearly 100% but was reduced to approximately 50% when *P. digitatum* inoculation was delayed until 3 d after wounding (Fig. 1A, lowercase letters). Fruit inoculated at 7 and 10 d after wounding did not show any rot development. At commercial harvest, decay incidence was not reduced to 50% until fruit were inoculated at 4 d after wounding and fruit inoculated at 7 and 10 d after wounding had decay incidences around 18%. Over-mature fruit showed a high degree of variability in disease incidence in response to elapsed time after wounding and thus no significant differences were observed. However, there was a reduction to approximately 50% when inoculation was done at 10 d after wounding.

No significant differences in decay incidence were found when fruit harvested at different maturity stages were inoculated at 0 h, 1, 2 and 3 d after wounding (Fig. 1A, uppercase letters). However, fruit from immature and commercial harvests inoculated at 7 and 10 d after wounding showed lower incidence (0 and 13%, respectively) than fruit from the over-mature harvest (67 and 56%, respectively). Statistical analysis revealed that, for each harvest time, lesion diameter significantly decreased with the elapsed time between wounding and inoculation (Fig. 1B, lowercase letters). Fruit from

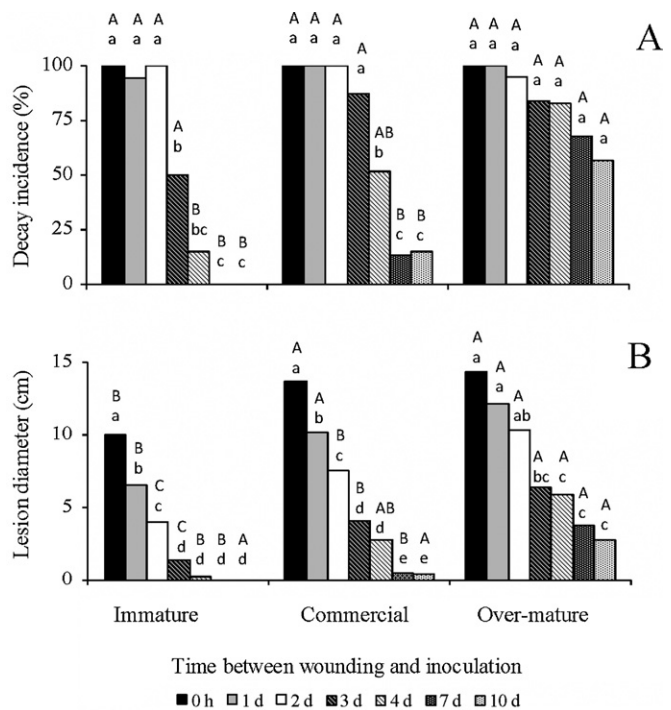


Fig. 1. Disease incidence (A) and lesion diameter (B) in 'Valencia' oranges harvested at three maturity stages and inoculated with *Penicillium digitatum* at different times after wounding and stored at 20 °C and 85% RH for 7 d. Disease incidence was transformed to the arcsine of the square root before analysis of data. For each harvest, lowercase letters indicate significant differences among inoculation times after wounding according to Tukey test ($P < 0.05$). For each inoculation time after wounding, harvests with different uppercase letters are significantly different according to Tukey test ($P < 0.05$). Each column represents the mean of 20 oranges.

the immature harvest inoculated at 0 h after wounding showed the largest lesion diameter (around 10 cm) in comparison to fruit inoculated at 1, 2, 3, 4, 7 and 10 d after wounding (6, 4, 1.5, 0.1, 0 and 0 cm, respectively). At commercial harvest, the effect of the elapsed time after wounding on lesion diameter showed five statistically different groups (0 h, 1 d, 2 d, 3–4 d, and 7–10 d after wounding) with lesion diameters around 13.5, 10, 7.5, 3.8 and 0.3 cm, respectively. Fruit from the over-mature harvest inoculated at 3, 4, 7 and 10 d after wounding showed smaller lesion diameters (6.3, 5.8, 3.7 and 2.7 cm, respectively) compared to those inoculated at 0 h after wounding (14.3 cm). While lesion diameters decreased with time after wounding, statistical classes overlapped and only the earliest and latest times of inoculation were statistically separated.

Fruit from the immature harvest inoculated at 0 h and 1 d after wounding (10 and 6.5 cm) showed smaller lesion diameters than those from the commercial harvest (13.6 and 10.1 cm) and those from the over-mature harvest (14.3 and 12.2 cm) (Fig. 1B, uppercase letters). However, when fruit were inoculated at 2 and 3 d after wounding, there were statistical differences in lesion diameter among fruit from all the three harvests. At 10 d after wounding no differences were observed between harvests.

3.1.2. At 4 °C

Decay incidence and lesion diameters in 'Valencia' oranges inoculated with *P. digitatum* at different times after wounding and incubated at 4 °C are shown in Fig. 2A and B, respectively. Data represent observations made at 30 d after inoculation. In general, observations made at 45 and 60 d after inoculation were difficult to impossible to assess because most of the oranges were completely rotten (data not shown).

In fruit from the immature harvest, decay incidence was approximately 60% when the inoculation was delayed to either 4 or 7 d

Table 2

Primer sequences, expected PCR product sizes (bp) and optimal annealing temperatures (°C) for genes used to select a reference gene for the expression studies.

Name	Sequence	Gene	GenBank accession no.	Annealing T (°C)	Amplicon (bp)
EF1F1 EF1R1	TGGTTTGTGCTTCCAACCTC CTTCAAGAAGCTGGGCTCCTT	Elongation factor 1 alpha	FC931731	60	220
GPDF1 GPDR1	GACTTGAGAAGGAGGCGACTT ACCCCATTCATTGTCATACCA	Glyceraldehyde-3- phosphate dehydrogenase	FC919596	60	215
ACTF1 ACTR1	ATGTGGATTGCCAAATCTGAG TTGTCCACACGTTGAAATGAA	Actin 11	CX298363	60	206
18SF1 18SR1	GTGACGGAGAATTAGGGTTCG CTGCCTTCCTGGATGTGGTA	18S rRNA	AF206997	60	70

after wounding (Fig. 2A, lowercase letters). Disease incidence in fruit inoculated at time 0 h after wounding was nearly 100%, therefore the delay in inoculation represented approximately a 40% reduction in disease incidence. When fruit were inoculated at 14 d after wounding, decay incidence was approximately 15% representing a reduction of approximately 85%. In fruit of the commercial harvest, no differences in decay incidence were found in fruit inoculated at 0 h and 4 d after wounding. In contrast, when inoculation was delayed 7 and 14 d, decay incidence was significantly reduced by approximately 30 and 60%, respectively. No rot development was found in fruit from either the immature or commercial harvests when they were inoculated at 21 d after wounding. In fruit from the over-mature harvest, a high degree of variability was observed in disease incidence in response to elapsed time after wounding and thus no significant differences were observed. However, there was a distinct tendency for the later inoculation times to exhibit reduced incidence since fruit inoculated at 0 h, 4 or 7 d after wounding showed 100% decay incidence and those inoculated at 14 or 21 d after wounding showed 70 and 50% decay incidence, respectively.

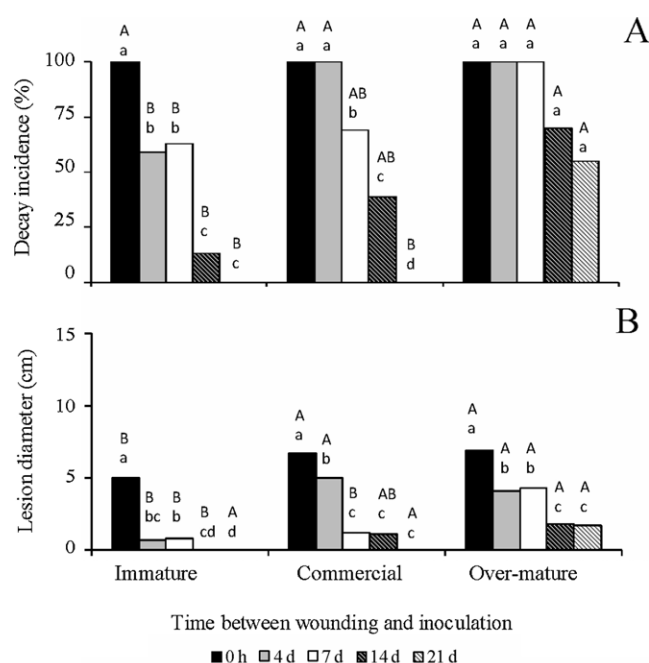


Fig. 2. Disease incidence (A) and lesion diameter (B) in 'Valencia' oranges harvested at three maturity stages and inoculated with *Penicillium digitatum* at different times after wounding and stored at 4 °C and 85% RH for 30 d. Disease incidence was transformed to the arcsine of the square root before analysis of data. For each harvest, lowercase letters indicate significant differences among inoculation times after wounding according to Tukey test ($P < 0.05$). For each inoculation time after wounding, harvests with different uppercase letters are significantly different according to Tukey test ($P < 0.05$). Each column represents the mean of 20 oranges.

A comparison among fruit from different harvests showed no significant differences in decay incidence when they were inoculated at 0 h after wounding (Fig. 2A, uppercase letters). However, decay incidence in fruit from the immature harvest was significantly lower at 4 d after wounding (approx. 60%) compared to fruit collected from the commercial and over-mature harvests (100%). When fruit were inoculated at 7 and 14 d after wounding, those from the over-mature harvest showed higher decay incidence (100 and 60%, respectively) compared to those from the immature harvest (65 and 15%, respectively) and when fruit were inoculated at 21 d after wounding, those fruit from the over-mature harvest had a higher decay incidence (50%) than those from the other two harvests (0%).

Statistical analysis indicated that within each harvest time, lesion diameter decreased as elapsed time between wounding and inoculation increased (Fig. 2B, lowercase letters). Fruit from the immature harvest inoculated at 0 h after wounding showed the largest lesion diameter (around 5 cm) in comparison to fruit inoculated at 4, 7, 14 and 21 d after wounding (<1 cm). At commercial harvest, the effect of elapsed time after wounding exhibited three statistically different groups (0 h, 4 d, and 7–21 d after wounding) with approximate lesion diameters of 6, 5 and 1 cm, respectively. However, no rot development was observed in fruit from either the immature or commercial harvests when they were inoculated at 21 d after wounding. In fruit from the over-mature harvest, the effect of elapsed time after wounding also resulted in three statistically distinct groups (0 h, 4–7 d, and 14–21 d after wounding) with lesion diameters around 7, 4 and 2 cm, respectively.

As shown in Fig. 2B (Fig. 2B, uppercase letters), fruit from the immature harvest inoculated at 0 h and 4 d after wounding had smaller lesion diameters (5 and 0.6 cm, respectively) than those from either the commercial (6.7 and 4.2 cm, respectively) or over-mature harvests (6 and 5 cm, respectively). Fruit from the immature harvest inoculated at 14 d after wounding were only statistically distinct from those the over-mature harvest. No significant differences were found among harvest dates and the elapsed time after wounding prior to inoculation was 21 d.

3.2. Effect of maturity stage and time between wounding and inoculation on development of mould caused by *P. expansum*

P. expansum inoculated at 0 h after wounding was able to develop rot at all harvests and temperatures assayed. Interestingly, decay incidence and lesion diameters obtained at 4 °C were higher than those obtained at 20 °C. In general, the elapsed time after wounding had a significant effect on restricting *P. expansum* infection and the effect was more pronounced in fruit from the immature and commercial harvests.

3.2.1. At 20 °C

P. expansum rot developed very slowly at 20 °C and after 4 and 7 d post-inoculation most of the oranges did not exhibit decay symptoms (data not shown). Therefore, decay incidence and lesion

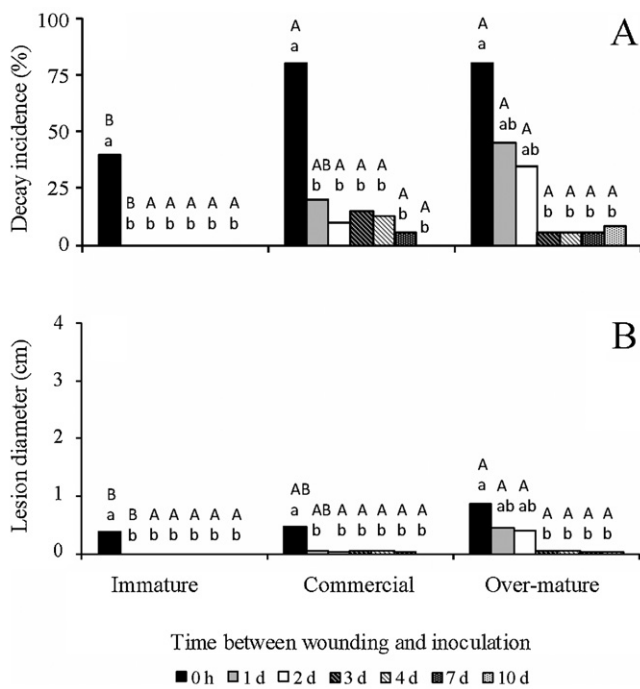


Fig. 3. Disease incidence (A) and lesion diameter (B) in 'Valencia' oranges harvested at three maturity stages and inoculated with *Penicillium expansum* at different times after wounding and stored at 20 °C and 85% RH for 10 d. Disease incidence was transformed to the arcsine of the square root before analysis of data. For each harvest, lowercase letters indicate significant differences among inoculation times after wounding according to Tukey test ($P < 0.05$). For each inoculation time after wounding, harvests with different uppercase letters are significantly different according to Tukey test ($P < 0.05$). Each column represents the mean of 20 oranges.

diameters in 'Valencia' oranges inoculated with *P. expansum* at different times after wounding and incubated at 20 °C are shown at 10 d after inoculation (Fig. 3A and B).

In fruit from the immature harvest, decay was completely absent when inoculation was delayed 1 d or longer (Fig. 3A, lowercase letters). In fruit from the commercial harvest, decay incidence was reduced to approximately 5–20% at 1, 2, 3, 4, and 7 d after wounding compared to around 80% at 0 h, representing about an 85% reduction. No rot development was found in fruit inoculated at 10 d after wounding. Fruit from the over-mature harvest showed rot development at all inoculation times. However, an 85% reduction in disease incidence was found when inoculation was delayed 3 d or longer after wounding.

The comparison among fruit from different harvests showed the most significant differences in decay incidence when fruit were wounded and inoculated at time 0 h (Fig. 3A, uppercase letters). Decay incidence was significantly lower in fruit from the immature harvest (40%) compared to fruit from the commercial and over-mature harvests (around 80%). Fruit from the immature harvest inoculated at 1 d after wounding also showed lower decay incidence (0%) compared to fruit from the over-mature harvest (40%). No significant differences in decay incidence were found when fruit harvested at different maturity stages were inoculated at 2, 3, 4, 7 and 10 d after wounding.

Significant differences in lesion diameter were found when fruit harvested at different maturity stages were inoculated at different times after wounding (Fig. 3B, lowercase letters). *P. expansum* only was able to develop rot in fruit from the immature harvest when they were inoculated at 0 h after wounding (0.4 cm). Fruit from the commercial harvest inoculated at 0 h after wounding had larger lesion diameters (0.5 cm) compared to those inoculated at 1, 2, 3, 4, 7 and 10 d after wounding (around 0.1 cm), whereas fruit inoculated

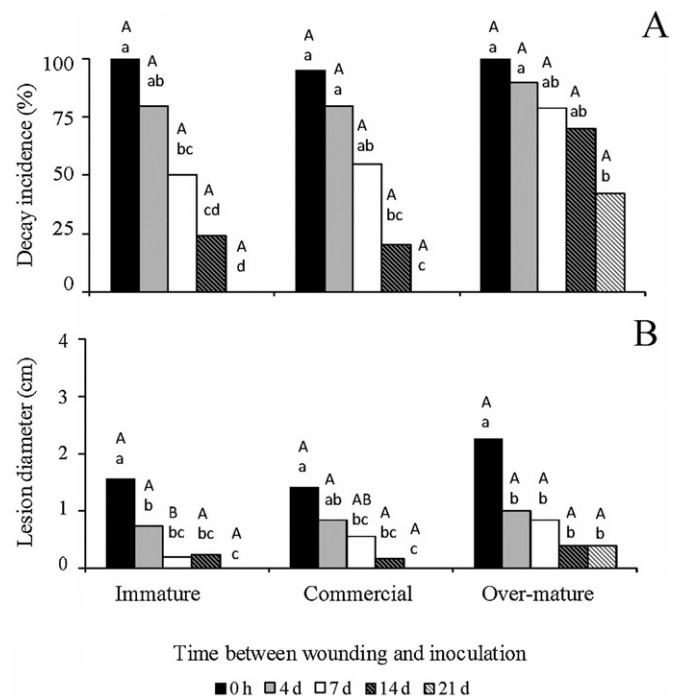


Fig. 4. Disease incidence (A) and lesion diameter (B) in 'Valencia' oranges harvested at three maturity stages and inoculated with *Penicillium expansum* at different times after wounding and stored at 4 °C and 85% RH for 45 d. Disease incidence was transformed to the arcsine of the square root before analysis of data. For each harvest, lowercase letters indicate significant differences among inoculation times after wounding according to Tukey test ($P < 0.05$). For each inoculation time after wounding, harvests with different uppercase letters are significantly different according to Tukey test ($P < 0.05$). Each column represents the mean of 20 oranges.

10 d after wounding did not show any disease incidence. Fruit from the over-mature harvest inoculated at 0 h after wounding showed larger lesion diameters (0.8 cm) compared to those inoculated at 3, 4, 7 and 10 d after wounding (around 0.1). Additionally, at this harvest, rot development was observed in fruit inoculated at all time points after wounding.

Fruit from the immature harvest inoculated at 0 h and 1 d after wounding had smaller lesion diameters (0.4 and 0 cm, respectively) than those from the over-mature harvest (0.8 and 0.4 cm, respectively). No significant differences in lesion diameter were found when fruit harvested at different maturity stages were inoculated at 2, 3, 4, 7 and 10 d after wounding.

When oranges from all three maturity stages were inoculated with *P. expansum* at 0 h and 1 d after wounding, a peel reaction, was observed encompassing both the flavedo and albedo tissues. The reaction consisted of dead tissue and an orange-red-coloured circle around inoculated wounds. In contrast, when oranges were wounded but not inoculated, or *P. expansum* inoculation was delayed from 2 to 10 d after wounding, no reaction was observed (data not shown).

3.2.2. At 4 °C

Decay incidence and lesion diameters of 'Valencia' oranges inoculated with *P. expansum* at different times after wounding and incubated at 4 °C are shown in Fig. 4A and B. The data is for 45 d after inoculation. Observations at 30 d after inoculation indicated very little decay (data not shown), and at 60 d after inoculation were difficult to evaluate among times between wounding and inoculation because most of the oranges were rotten (data not shown).

In fruit from the immature harvest, decay incidence was reduced to 50 and 25%, compared to 100% at 0 h, when inoculation was delayed 7 and 14 d, respectively (Fig. 4A, lowercase letters). Similar

patterns were obtained in fruit from the commercial harvest. No rot development was observed in fruit from the immature and commercial harvests when they were inoculated at 21 d after wounding. In fruit from the mature harvest, decay incidence was reduced by approx. 70% when inoculation was delayed until 21 d after wounding.

No significant differences in decay incidence were observed when disease incidence for each specific inoculation time was compared between harvest groups (Fig. 4A).

Statistical analysis indicated that within each harvest group, lesion diameter decreases as the elapsed time between wounding and inoculation increased (Fig. 4B, lowercase letters).

Fruit from the immature harvest inoculated at 0 h after wounding had the largest lesion diameter (1.7 cm) compared to fruit inoculated at 4, 7, 14 and 21 d after wounding (lower than 1 cm). In general, a similar pattern was observed in fruit obtained from the commercial and immature harvests. Moreover, no rot development was found in fruit from the immature and commercial harvests when they were inoculated at 21 d after wounding. Fruit from the over-mature harvest had a larger lesion diameter when they were inoculated at 0 h after wounding (2.3 cm) than when they were inoculated at 4, 7, 14 and 21 d after wounding (lower than 1 cm).

In general, no significant differences in lesion diameter for specific inoculation times were found when each time point was compared among harvest groups (Fig. 4B, uppercase letters).

3.3. Quality parameters

Significant differences in 'Valencia' quality parameters were found between the harvest groups (Table 3). While total soluble solids (TSS) did not differ significantly between the harvest groups, titratable acidity (TA) and colour index (CI) decreased as the harvest date progressed. Accordingly, TSS/TA ratio was higher in the over-mature harvest group when compared to both the immature and commercial harvest groups. As a measure of fruit firmness, deformation of oranges increased when applying 2 kg of force as harvest date progressed. The increase was probably attributed to pectin degradation.

3.4. Lignin content

Table 4 shows the lignin content (absorbance at 280 nm) in 'Valencia' oranges tissue surrounding wounds inoculated with either water (control), *P. digitatum*, or *P. expansum* and stored at 20 °C for up to 7 d.

Differences in lignin content with time after inoculation were evident in the control treatment and in samples inoculated with *P. expansum* in fruit collected from the immature harvest (Table 4, lowercase letters). In both cases, lignin content was higher at 7 d after inoculation than at 24 and 48 h.

Differences in lignin content in control samples from the three different harvest groups appeared at 96 h and 7 d after inoculation. Samples inoculated with *P. expansum* only showed differences between harvests at 96 h after inoculation (Table 4, uppercase letters). The control treatment at 96 h after inoculation had higher lignin content in the immature harvest group than in the over-mature harvest group. The highest levels of lignin were observed in fruit from the immature harvest group at 7 d after wounding and wounding plus inoculation with *P. expansum*. Samples inoculated with *P. expansum* showed higher lignin content at 96 h in fruit from the immature harvest group than fruit at 96 h from the commercial harvest group. No differences between the immature and commercial harvest groups were found in samples inoculated with *P. digitatum* and stored for 24 h. Subsequent measurements were not possible because the fruit was completely rotten.

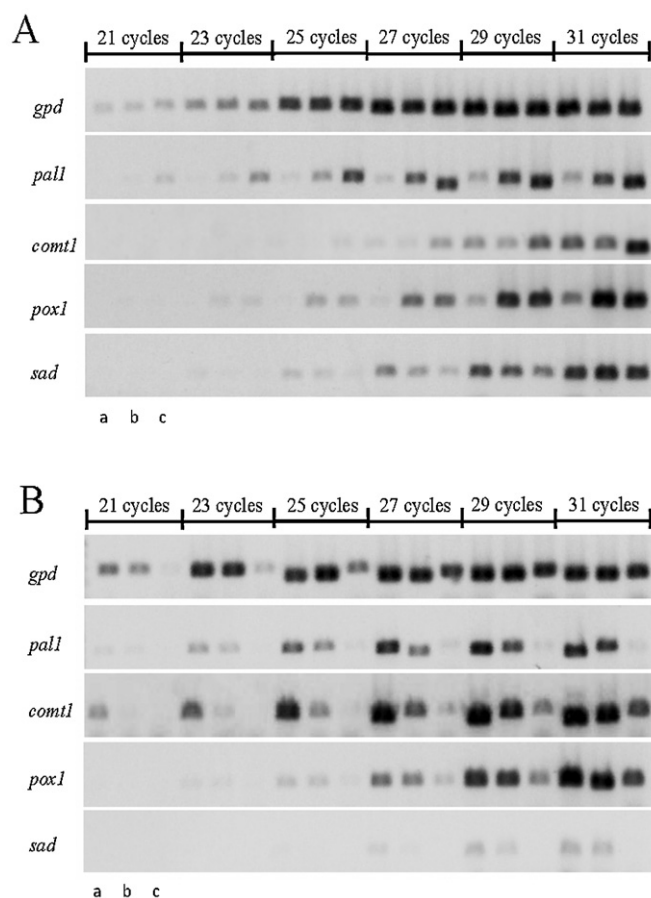


Fig. 5. Semi-quantitative RT-PCR analysis of the expression of several phenylpropanoid pathway-related genes (*pal1*, *comt1*, *pox1* and *sad*) in oranges wounded and inoculated with water (a), *Penicillium expansum* (b) and *Penicillium digitatum* (c), respectively, at each PCR cycle. Oranges were stored at 20 °C and 85% RH for 24 h (A) and 48 h (B). The *gpd* gene was used as a reference for normalizing mRNA quantity.

In summary, an increase in lignin content was observed over the 7 d period in control samples and those inoculated with *P. expansum*. The strongest lignin response was observed in fruit collected in the immature harvest group with much weaker responses in the later harvest groups, especially in the over-mature group where lignin response appeared to be insufficient to block or delay infection by *P. expansum*. Little lignin accumulation was observed in samples that were wounded and inoculated with *P. digitatum* regardless of the harvest date.

When the samples presented decay (independently if they were inoculated with *P. digitatum* or *P. expansum*) the absorbance values obtained at 280 nm as lignin content showed unusual high values (data not shown).

3.5. Gene expression of several phenylpropanoid pathway-related genes

The expression of several genes involved in the phenylpropanoid pathway was studied in oranges inoculated with *P. digitatum*, *P. expansum*, or water as a control (Fig. 5).

Semi-quantitative analysis revealed different expression profiles for each gene depending on the time after inoculation. At 24 h after inoculation, the accumulation of *pal1* mRNA was 4 and 16 times higher in samples infected with *P. expansum* and *P. digitatum*, respectively, than in control samples and the accumulation of *pox1* mRNA was 4 times higher in samples infected with both pathogens compared to control samples. *comt1* expression was

Table 3
Effect of harvest date on fruit quality parameters of 'Valencia' oranges. Values for harvest dates with the same letter are not significantly different ($P < 0.05$) according to the Tukey test.

Harvest	Date	Total soluble solids (TSS in %)	Titrateable acidity (TA in % citric acid)	Ratio TSS/TA	CI (colour index)	Deformation (mm)
1	20/03/2010	10.0 a	1.49 a	6.7 b	4.6 a	2.2 b
2	30/04/2010	9.9 a	1.10 b	9.0 b	3.9 b	2.5 ab
3	29/06/2010	11.3 a	0.85 c	13.3 a	3.1 c	2.7 a

induced around 4-fold only in fruit infected with *P. digitatum*. A different pattern was observed with *sad*, whose expression was highest in control fruit. At 48 h after inoculation *pal1*, *comt1*, *pox1* and *sad* showed a higher expression in control fruit than at 24 h after inoculation. However, the most noticeable difference between 24 and 48 h after inoculation was the decreased expression found in samples inoculated with *P. digitatum* at 48 h after inoculation. No differences were found for *cad2* between treatments or between times after inoculation (data not shown).

4. Discussion

Increasing innate resistance of fruit to fungal pathogens is one of the alternatives being explored in the effort to reduce the dependency on chemicals for postharvest disease control. Investigations have focused on inducing citrus resistance against *P. digitatum* using either curing (Brown et al., 1978; Brown and Barmore, 1983; Plaza et al., 2003; Ballester et al., 2010) or hot water treatments (Nafussi et al., 2001; Palou et al., 2001). However, little is known about how resistance in citrus is impacted by wound response and the effect of ripening and other factors on that response. The current study evaluated wound response in 'Valencia' oranges in relation to the ability of *P. digitatum* (pathogen) and *P. expansum* (non-host pathogen) to infect fruit at different maturity stages and storage conditions.

Results indicated that wound response had a clear effect on *P. digitatum* when fruit were stored at 20 °C. Significant decrease in both disease incidence and lesion diameter was found when inoculation was delayed 7 and 10 d after wounding. Baudoin and Eckert (1985) found decay incidences around 0 and 6% in green and yellow-green lemons, respectively, when they were inoculated with *P. digitatum* at 20 h after wounding and held at 25 °C and 100% RH for 5 d. Brown et al. (1978) obtained similar results with oranges at 72 h after inoculation. This study has demonstrated that time after inoculation is a very important factor when comparing the results of several studies. In pear, Spotts et al. (1998) found that wound healing decreased the susceptibility of wounds to *P. expansum* infection after 2 d at 20 °C. Wound response appears to

be more efficient in pears than in oranges in providing resistance to a compatible pathogen because only in 2 d they observe similar reductions than those observed in the present study in 4 d. On the other hand, the effect of wound response on resistance of green peppers to *Colletotrichum acutatum* was even faster because delaying inoculation for only 1 h after wounding resulted in a great reduction in lesion size (Kim et al., 2008).

The present study demonstrated that the effect of wound response on resistance is temperature dependent. Temperature and relative humidity are the two most important conditions affecting the process of wound healing process (Brown, 1989). Temperatures must be sufficiently high to encourage rapid development of the metabolic reactions involved in healing and adequate moisture must be present to prevent desiccation and death of the tissues surrounding the damaged cells. Generally, temperatures above 10 °C and a relative humidity above 85% are required for the wound healing process in citrus (Brown, 1989). Significant differences in lesion diameter among different times between wounding and inoculation were observed at 30 d after inoculation in fruit collected at a commercial harvest stage and stored at 4 °C (Fig. 2). However, at 45 d after inoculation, it was not possible to evaluate differences among times between wounding and inoculation because the oranges were completely rotten (15 cm lesion diameter). This indicates that wound response at cold temperatures was insufficient in preventing colonization of *P. digitatum*. Vilanova et al. (2012) obtained similar results in oranges inoculated with the non-pathogen *P. expansum*. However, Lakshminarayana et al. (1987) observed a strong resistance in apples inoculated with *Botrytis cinerea* and *P. expansum* within 4 d after wounding at 5 °C. They considered that 4 d between wounding and inoculation was not enough time to produce modifications in the cell wall at 5 °C and for this reason they attributed the resistance response to processes other than wound healing. Spotts et al. (1998) reported that pears stored at –1 °C and inoculated 28 d after wounding decreased decay incidence from 93% to 35%.

Maturity stage appears to be an important factor in determining the resistance of oranges to the host-specific pathogen, *P. digitatum*, and non-host pathogens such as *P. expansum* (Vilanova et al., 2012).

Table 4
Lignin content (absorbance at 280 nm) of 'Valencia' oranges wounded and inoculated with water (control), *P. digitatum*, or *P. expansum* and stored at 20 °C for different periods of time. Oranges were harvested at three different maturity stages. For each harvest, times after inoculation with different lowercase letters are statistically different according to the Tukey test ($P < 0.05$). For each time after inoculation, harvests with different uppercase letters are statistically different according to Tukey test ($P < 0.05$).

Harvest	Time after inoculation	Lignin content (absorbance at 280 nm)		
		Control	<i>P. expansum</i>	<i>P. digitatum</i>
Immature	24 h	0.5028 b A	0.4577 c A	0.3870 A
	48 h	0.5092 b A	0.5411 bc A	Rot develop
	96 h	0.5877 ab A	0.6288 ba A	Rot develop
	7 d	0.6199 a A	0.6741 a	Rot develop
Commercial	24 h	0.4269 a A	0.4147 a A	0.3932 A
	48 h	0.4582 a A	0.4117 a A	Rot develop
	96 h	0.4806 a AB	0.5044 a B	Rot develop
	7 d	0.4567 a B	Rot develop	Rot develop
Over-mature	24 h	0.4538 a A	Rot develop	Rot develop
	48 h	0.4066 a A	Rot develop	Rot develop
	96 h	0.4471 a B	Rot develop	Rot develop
	7 d	0.4677 a B	Rot develop	Rot develop

This was confirmed in the present study since the most significant differences among times between wounding and inoculation at both assayed temperatures were observed in fruit from the immature and commercial harvests. Similar differences were observed in light green and yellow lemons (Baudoin and Eckert, 1985). In apples, Su et al. (2011) reported that *B. cinerea* decay severity (lesion size) increased significantly in more mature fruit after wounding and delayed inoculation but that decay incidence in wounds that were immediately inoculated was similar in fruit harvested at all harvest dates.

Ripening is a process that involves numerous biochemical changes such as cell wall disassembly and cell membrane alteration (Cantu et al., 2008a). Therefore, the ripening process can potentially increase fruit susceptibility of wounds to fungal infection due a reduced wound defence response. Even though citrus peel is considered an inappropriate and even toxic environment for germination and growth of non-host pathogens (Stange et al., 2002), different authors have demonstrated that the non-host pathogen, *P. expansum*, can infect citrus fruit under some conditions (Macarasin et al., 2007; Vilanova et al., 2012). In the present study, *P. expansum* was able to grow even at different times between wounding and inoculation. However, at 20 °C, the lesion diameter obtained in all cases was less than 1 cm. In contrast, decay incidence and severity were greater at 4 °C. Vilanova et al. (2012) reported the same observation and suggested that wound healing is slower at cold temperatures and that *P. expansum* is well adapted to these colder temperatures (Gougouli and Koutsoumanis, 2010).

Different authors have correlated wound healing process in oranges with lignin accumulation (Ismail and Brown, 1975; Brown et al., 1978; Brown and Barmore, 1983; Vilanova et al., 2012). Most of these studies used histochemical techniques to analyze lignin deposition in the orange tissues. Nafussi et al. (2001), however, used a quantitative assay to measure lignin content in inoculated orange wounds which we adopted in the present study. Our results showed that only wounded fruit from the immature harvest increased in lignin content with storage time. Control samples had the highest lignin quantity at 7 d after wounding. Similar results were reported by Su et al. (2011) in apples where a greater increase in lignin was observed in wounded tissue from early harvested fruit compared to late harvest fruit. These results are in agreement with those obtained in the present study in which fruit from the immature harvest did not exhibit *P. digitatum* decay when the inoculation was done 7 d after wounding which could be related to lignin accumulation. However, Vilanova et al. (2012) using a histochemical stain did not find a positive lignin reaction in orange fruit that has been just wounded and not inoculated.

In immature oranges inoculated with *P. expansum* and stored at 20 °C, lignin content also increased over a 7 d period. Similar results were obtained in an earlier study using a histochemical stain for lignin where the strongest lignin reaction was observed in immature fruit at 7 d after inoculation.

Nafussi et al. (2001) found that lignin content did not increase, and in some cases decreased, at 4 d after inoculation in lemons inoculated with *P. digitatum*. In our case, when the pathogens were able to develop rot an anomalous high value in lignin content was obtained using the quantitative assay of Nafussi et al. (2001). The increase in absorbance with the acetyl bromide method could be attributed to an increase in polysaccharide degradation and suggests that factors other than the solubilization of lignin were responsible for the increase in absorbance (Hatfield et al., 1999). When necrotrophic pathogens infect fruit, they secrete a substantial array of cell wall degrading enzymes that target a variety of plant cell wall polysaccharides and causes extensive tissue destruction (Cantu et al., 2008b). This modification in the orange cell wall can result in a significant increase in polysaccharides that may interfere in lignin quantification. Therefore, the use

of the acetyl bromide method to quantify lignin should be used cautiously when cell wall degradation is taking place since erroneous absorbance values may be obtained. Numerous methods have been developed over the past years to measure lignin levels in different plant species (Hatfield and Fukushima, 2005) however, choosing the most suitable method for each fruit remains a difficult task.

Gene expression analysis revealed that the phenylpropanoid pathway is associated with wound response (abiotic) and pathogen challenge (biotic) in citrus. A previous study in citrus fruit focused on changes in phenylpropanoid-related gene expression in response to a compatible pathogen (Ballester et al., 2011) but until the present study, changes in response to non-host pathogen remained unexplored. PAL is involved in the first step of the phenylpropanoid pathway, which is responsible for the production of lignin (Dixon et al., 2002). In our study, the accumulation of *pal1* mRNA was 4 and 16 times higher at 24 h after inoculation in samples infected with *P. expansum* and *P. digitatum*, respectively, compared to control samples (only wounded) and the accumulation of *pox1* mRNA in samples infected with both pathogens was 4 times higher than in control samples. These results suggest that these genes are specifically induced to high levels in response to both compatible and non-host pathogens. However, at 48 h the expression profile of *pal1*, *comt1*, *pox1* and *sad* greatly increased in response to wounding and decreased dramatically in response to *P. digitatum*. These results indicate that orange response to the wounding is slower than the response to pathogen attack. Ballester et al. (2013) also found that in wounded oranges *pal1*, *comt1* and *pox1* had maximum expression levels at 48–72 h. The decrease in the expression level of *pal1*, *comt1*, *pox1* and *sad* at 48 h in response to *P. digitatum* indicates that this compatible pathogen is able to suppress the expression of several genes involved in the phenylpropanoid pathway and thereby enhance infection. A similar decrease in expression was found by Ballester et al. (2013) at 72 h in response to *P. digitatum*. This difference in the timing of the decrease reported in Ballester et al. (2013) and the present study could be due to *P. digitatum* inoculum concentration used in each study. The higher concentration used in our study could result in an earlier decrease in gene expression.

Our study provides a pathological, biochemical and molecular approach to the characterization of wound response in orange. Data indicate that the wound response process at 20 °C can prevent infection by both compatible, *P. digitatum*, and non-host, *P. expansum*, pathogens. However at cold temperatures, wound response was too slow to prevent infection by the same pathogens. Additionally, immature fruit produced higher lignin content and were more resistant than commercial and over-mature fruit. Lastly, gene expression results indicate that *P. digitatum* can suppress the expression of several genes involved in the phenylpropanoid pathway and thereby enhance infection development. This study provides information on how wound response in citrus can be affected by both maturity stage and storage conditions (temperature). Such information can be used to determine the best time to apply antifungal products and help to design management practices that support resistance and reduce fruit rot.

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